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MOLECULAR PATHOGENESIS

Bile Salts Regulate Zinc Uptake and Capsule Synthesis in a Mastitis-Associated Extraintestinal Pathogenic *Escherichia coli* Strain

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ABSTRACT Extraintestinal pathogenic *Escherichia coli* (ExPEC) strains are major causes of urinary and bloodstream infections. ExPEC reservoirs are not completely understood. Some mastitis-associated *E. coli* (MAEC) strains carry genes associated with ExPEC virulence, including metal scavenging, immune avoidance, and host attachment functions. In this study, we investigated the role of the high-affinity zinc uptake (*znuABC*) system in the MAEC strain M12. Elimination of *znuABC* moderately decreased fitness during mouse mammary gland infections. The Δ*znuABC* mutant strain exhibited an unexpected growth delay in the presence of bile salts, which was alleviated by the addition of excess zinc. We isolated suppressor mutants with improved growth in bile salts, several of which no longer produced the K96 capsule made by strain M12. The addition of bile salts also reduced capsule production by strain M12 and ExPEC strain CP9, suggesting that capsule synthesis may be detrimental when bile salts are present. To better understand the role of the capsule, we compared the virulence of mastitis strain M12 with that of its unencapsulated Δ*kpsCS* mutant in two models of ExPEC disease. The wild-type strain successfully colonized mouse bladders and kidneys and was highly virulent in intraperitoneal infections. Conversely, the Δ*kpsCS* mutant was unable to colonize kidneys and was unable to cause sepsis. These results demonstrate that some MAEC strains may be capable of causing human ExPEC illness. The virulence of strain M12 in these infections is dependent on its capsule. However, capsule may interfere with zinc homeostasis in the presence of bile salts while in the digestive tract.

KEYWORDS zinc transport, group III capsule, mastitis-associated *E. coli*, ExPEC, bile salts, ZnuABC

Escherichia coli strains are abundant members of the healthy intestinal flora of most mammals and birds and may also be obligate or opportunistic pathogens. Strains that reside in the digestive tract but cause disease in other tissues are termed extraintestinal pathogenic *E. coli* (ExPEC) (1). ExPEC strains are a major cause of urinary tract infections, neonatal meningitis, pneumonia, and sepsis in humans. ExPEC strains also cause several diseases of agricultural importance, including airway infections and septicemia in poultry (2). Avian pathogenic strains present in poultry products represent a significant risk for human infection (3).

ExPEC strains are not derived from a single lineage but rather arise from frequent recombination within diverse phylogenetic backgrounds (4). Furthermore, there are no specific virulence genes that are universally present in all ExPEC strains, although many genes that are common in pathogenic strains have demonstrated roles in experimental infections. Among these are genes that enable the bacteria to attach to and invade their hosts, resist the antimicrobial effects of serum, scavenge for metal ions, or produce toxins (5).

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E. coli strains are also the most frequent cause of bovine mastitis, which is responsible for billions of dollars in losses annually to dairy producers (6). The strains that cause mastitis (mastitis-associated *E. coli* [MAEC]) have traditionally been viewed as commensals that cause disease solely by triggering inflammation in the mammary gland rather than as pathogens (7). As with ExPEC, there is not a reliable genetic marker that distinguishes MAEC from other *E. coli* strains. Several recent comparative genomic studies of MAEC strains have highlighted the diversity of these bacteria (8–12). They have also noted that some MAEC strains carry genes associated with the virulence of ExPEC strains (12–14). The function of these ExPEC virulence genes in MAEC has not been investigated. As these genes are also commonly found in commensal strains that are not known to cause extraintestinal disease, their purpose may be to facilitate gastrointestinal colonization and persistence (15, 16). These genes could also increase bacterial survival and tissue damage during mammary gland (MG) infection by some strains. Finally, it is also possible that some MAEC strains are fully capable of causing ExPEC-like disease, and pathogenesis in extraintestinal sites depends on these virulence-associated genes.

We previously conducted a genome-wide transposon sequencing (TnSeq) screen to identify fitness factors needed by an MAEC strain (M12) to grow in milk and colonize mouse MGs (12). We demonstrated a role for an individual MAEC gene in mastitis for the first time. An M12 mutant lacking the *fecA* gene was unable to grow in milk and was attenuated during MG infection. The *fecA* gene encodes the ferric dicitrate receptor and is enriched among MAEC strains (17), but it is not known to contribute to other ExPEC diseases. ExPEC-associated virulence genes were also identified as potentially contributing to MG colonization. These included genes for group III capsule biosynthesis identical to those of the K96 serotype (18). Elimination of the *kpsCS* capsule genes from strain M12 resulted in a modest decrease in fitness in mouse MG colonization. The TnSeq screen also suggested that the high-affinity zinc uptake system encoded by the *znuABC* genes may be important for colonizing MGs.

Between 5 and 6% of all *E. coli* proteins may require zinc binding for proper function (19), making it an essential nutrient for bacterial growth. Since free zinc is limited in host tissues, bacterial pathogens must scavenge for zinc to overcome defenses that are part of nutritional immunity (20). One important host zinc sequestration mechanism is the release of calprotectin by neutrophils, which binds to zinc as well as other metals (21). Infectious bacteria can scavenge for zinc by releasing zincophores (22, 23) or through high-affinity transport systems such as the Znu/Adc family (24–27). The periplasmic zinc-binding protein ZnuA, the integral membrane subunit ZnuB, and the ATP-binding subunit ZnuC actively import scarce zinc ions across the inner membrane. Zinc scavenging via the Znu system enhances the virulence of several bacterial pathogens, including ExPEC (28–33), but its role in MG colonization is unknown. High numbers of neutrophils infiltrate infected MGs during mastitis, so it is possible that the expression of the Znu proteins benefits MAEC strains in this environment.

In this study, we investigated the role of the ExPEC virulence-associated *znuABC* genes in the MAEC M12 strain. We show that an M12 Δ*znuABC* mutant has decreased survival in mouse MGs. We also observed that the M12 Δ*znuABC* mutant has a pronounced growth delay when exposed to bile salts. Further examination of this growth defect uncovered a link between the presence of bile salts, zinc uptake, and capsule production, where capsule appears to limit bacterial growth when bile salts are present. Conversely, we demonstrate that capsule synthesis is required for strain M12 to cause ExPEC-like disease, including urinary tract infection and sepsis.

RESULTS

Role of *znuABC* in colonization of mouse mammary glands. Our previous TnSeq analysis suggested that the high-affinity zinc transport system (*znuABC*) is essential for MAEC strain M12 to colonize mouse MGs (12). The *znuABC* locus contains two divergently transcribed units: *znuCB* are transcribed together, while *znuA* is transcribed

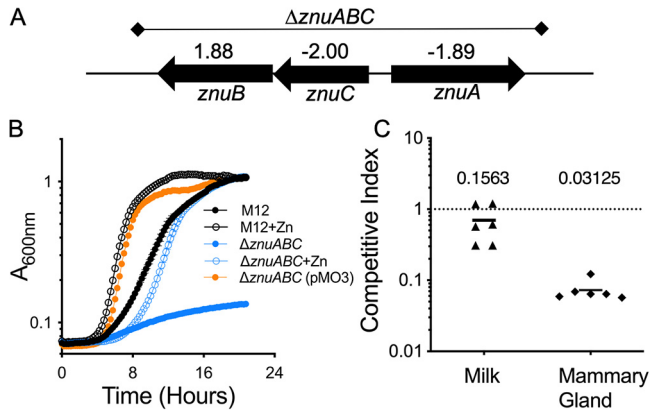

FIG 1 Role of the *znuABC* zinc transporter in the mastitis-associated *E. coli* M12 strain. (A) The *znu* locus of strain M12 and the 2,560-bp region that was deleted to create the Δ*znuABC* mutant strain. The normalized TnSeq fitness scores predicted for each gene as calculated by Rendon et al. (34) are indicated. (B) Growth of the M12 wild-type, Δ*znuABC* mutant, and Δ*znuABC*(pMO3) complemented strain in LB broth containing 1.0 mM EDTA (means ± standard errors of the means [SEM]) ($n = 3$). The mutant displayed a significant delay in growth that was fully restored with the addition of 100 μM zinc or by complementation with the plasmid pMO3. The addition of supplemental zinc enhanced the growth of the wild-type strain. (C) Competitive fitness of the Δ*znuABC* mutant compared with the M12 wild-type strain during growth in milk ($n = 6$) or in mouse mammary glands ($n = 6$). Equal ratios of both strains were inoculated in unpasteurized bovine milk or injected through the teat canal of lactating mice. Bacterial numbers were determined after 8 h and 24 h, respectively. The mutant strain was significantly less fit (*, $P = 0.03125$ by a Mann-Whitney test) than the wild-type strain in mammary glands but not milk.

divergently (Fig. 1A). Reexamination of our TnSeq data suggested that transposon (Tn) insertions in *znuA* or *znuC* but not *znuB* are detrimental to M12 colonization of MGs (34). In order to ascertain the importance of this system, we chose to delete the entire *znuABC* gene cluster from strain M12. To confirm that the Δ*znuABC* mutant strain was sensitive to zinc-limiting conditions, the mutant and wild-type M12 strains were cultured in the presence of the divalent cation chelator EDTA. Growth of the Δ*znuABC* mutant was suppressed by the addition of 1.0 mM EDTA (Fig. 1B). Complementation via a low-copy-number plasmid enhanced the ability of the mutant to grow in EDTA-containing medium. Growth of the Δ*znuABC* mutant strain also resembled that of the wild type when supplemental zinc (1 mM) was added to the medium with EDTA. Manganese and copper did not restore the growth of the Δ*znuABC* mutant, even up to concentrations that slowed the growth of the wild-type strain (see Fig. S1 in the supplemental material). These results demonstrate that the M12 Δ*znuABC* mutant is specifically deficient in its ability to acquire zinc. We then tested the fitness of the mutant strain under conditions relevant to mastitis, including growth in milk and during infection of lactating mouse MGs. Competition assays demonstrated that the loss of *znuABC* did not significantly affect fitness during growth in milk, but it resulted in an approximately 10-fold competitive disadvantage compared to wild-type bacteria in mouse MGs (Fig. 1C).

Effect of *znuABC* on growth in the presence of bile salts. In these experiments, the competing strains were selected on MacConkey agar plates after growth in milk or mammary glands. We observed that when the Δ*znuABC* mutant was grown on MacConkey agar plates, colonies took 3 to 4 days to appear, but when grown on Luria-Bertani (LB) agar, it grew at the same rate as the wild-type strain. Bile salts and crystal violet are the primary constituents of MacConkey agar that select for Gram-negative enteric bacteria. In order to determine what was responsible for the delayed growth of the mutant strain, we tested growth in LB medium containing bile salts or crystal violet. While crystal violet had no effect, the Δ*znuABC* mutant exhibited a delayed-growth phenotype in medium containing as little as 0.5% bile salts (Fig. 2A). The total growth yield was the same for both wild-type and mutant bacteria. The growth rate during

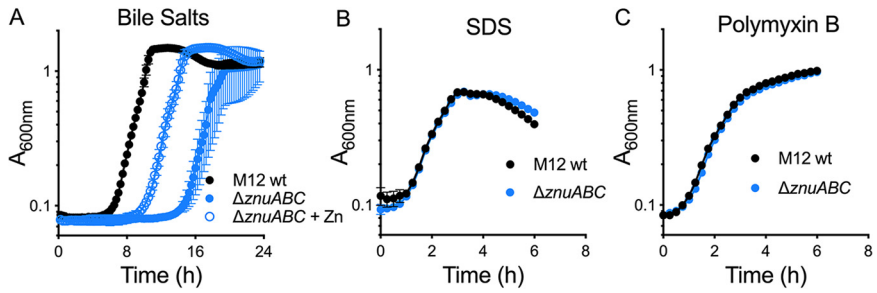

FIG 2 Loss of *znuABC* delays the growth of strain M12 specifically in the presence of bile salts. (A) Growth of M12 or the Δ*znuABC* mutant in LB medium containing 2% bile salts (means ± SEM) ($n = 3$). The mutant strain entered the exponential growth phase several hours later than the wild type (wt) but had similar absorbances at 600 nm and during the saturation phase. The addition of 100 μM zinc to the medium decreased the time that it took for the Δ*znuABC* mutant to enter exponential phase. (B and C) Identical growth curves of wild-type M12 and the Δ*znuABC* mutant in 5% SDS (B) or 2 μg/ml of polymyxin E (C) showing that the mutation does not confer a generalized membrane defect.

exponential phase appeared to be the same, but the mutant did not enter the exponential growth phase until 12 to 14 h after inoculation.

Bile salts have detergent qualities that damage bacterial membranes. To determine if the loss of *znuABC* causes a generalized membrane defect, we investigated whether the mutant strain had attenuated or delayed growth in SDS detergent or polymyxin B antibiotic, both of which can disrupt membrane integrity. The Δ*znuABC* mutant did not have a growth defect in either SDS or polymyxin (Fig. 2B and C). As the Δ*znuABC* mutant does not have a generalized membrane defect, our results suggest a novel role for bile resistance that is dependent on zinc utilization. In support of this interpretation, the addition of supplemental zinc (1 mM) to the medium with bile salts decreased the time that it took for the Δ*znuABC* mutant to enter exponential phase (Fig. 2A).

A suppressor mutant screen suggests a link between K96 capsule and growth in bile salts. The Δ*znuABC* mutant grew to the same density as the wild-type strain after 24 h. We wanted to determine if these bacteria that had grown in the bile salt medium would exhibit the same growth delay when subcultured in the same medium. Transfer of these bacteria directly into LB medium with bile salts showed that they entered exponential phase at the same time as the wild-type strain. This suggested that the mutant strain adapted to the bile salts, through either changes in gene expression, compensatory mutations, or both. We then plated the bile-salt-adapted mutant strain on LB agar without bile salts to obtain single colonies. These colonies were then cultured in broth with bile salts (Fig. 3A) to see if the lag in growth reappeared. The "adapted" mutants grew at the same rate as the wild-type strain, suggesting that suppressor mutants readily arose among the Δ*znuABC* mutant population in the presence of bile salts.

To identify the genetic basis for these suppressor mutations, we serially passaged 10 separate cultures derived from the Δ*znuABC* parent strain in the presence of 2% bile salts for approximately 200 generations. From these cultures, we isolated individual colonies on LB agar plates and then tested their growth profiles in bile salts compared to the wild-type or Δ*znuABC* strains (Fig. 3B). All of the colonies that we isolated grew more rapidly than the wild-type strain in LB medium with bile salts. The genomes of the 10 suppressor mutants as well as the Δ*znuABC* mutant parent strain were sequenced, and nonsynonymous single nucleotide polymorphisms (SNPs) within predicted coding regions were identified (Table 1). Three of the mutants had nucleotide substitutions in the *rpoA* gene encoding the alpha-subunit of RNA polymerase. All three mutations are predicted to change asparagine 294 to a histidine or lysine. Four mutants contained SNPs in the *dedD* or *ftsK* gene, which are predicted to result in nonfunctional alleles, either eliminating start codons or introducing premature stop codons. Both DedD and FtsK are proteins involved in cell division. Finally, three suppressor mutants contained

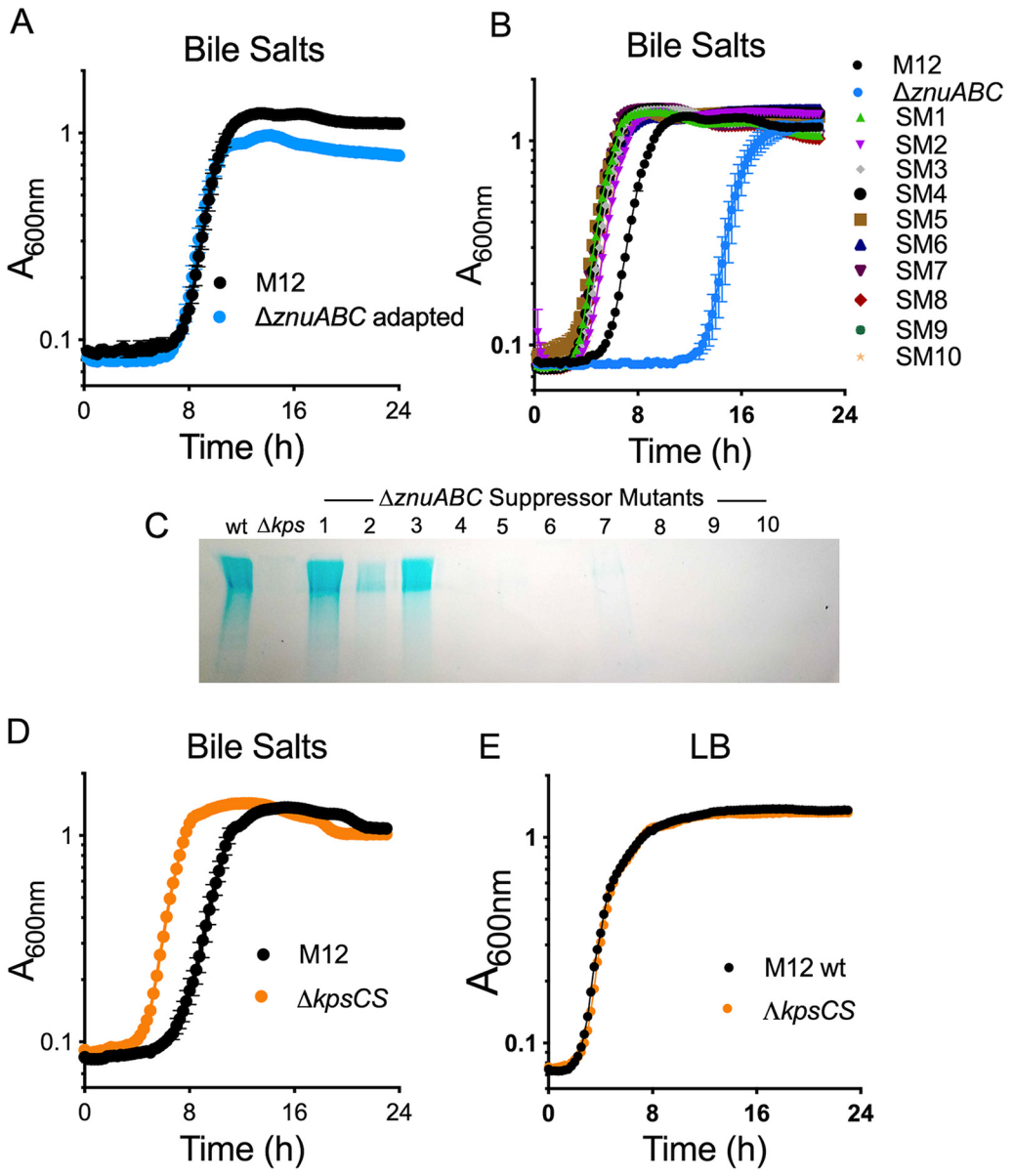

FIG 3 Suppressor mutations in the Δ*znuABC* background that restore growth in bile salts also eliminate capsule synthesis. (A) Saturated cultures of the wild-type strain and the Δ*znuABC* mutant grown in LB medium with bile salts and subcultured in the same medium exhibited similar growth (means ± SEM) ($n = 3$), suggesting the possibility of suppressor mutations arising in the population. (B) Ten independently derived suppressor mutants had increased growth in LB medium containing 2% bile salts compared to the wild-type strain. Whole-genome sequencing indicated that suppressor mutants 2, 8, and 10 contained SNPs that mapped to predicted capsule synthesis genes. (C) Gel electrophoresis and alcian blue staining (image representative of an experiment performed three times) showing that 8 of 10 suppressor mutants produce less capsule than the wild-type strain. (D and E) A Δ*kpsCS* mutant unable to produce capsule reaches exponential phase faster than the wild-type strain when grown in LB medium with bile salts, but growth in LB medium is indistinguishable from that of the wild-type strain.

SNPs in glycosyltransferase genes found in a cluster associated with biosynthesis and export of a K96 group III capsule.

These results suggested a link between capsule production and growth in bile salts. Therefore, we sought to determine whether these glycosyltransferase gene mutations altered capsule production. We also wanted to determine whether the other suppressor mutants produce capsule normally. We compared capsule synthesis of the M12 wild type, an unencapsulated Δ*kpsCS* mutant (12), and each of the suppressor mutant strains (Fig. 3C) using SDS-PAGE and alcian blue staining. The suppressor mutants with

TABLE 1 SNPs identified in Δ*znuABC* suppressor mutants with enhanced growth in bile salts

Strain	Gene	Mutation	Function
SM1	*ftsK*	212_213insTGGCAGA	Chromosome segregation in cell division
SM2	CO715_12230	E45Stop	Capsule synthesis, glycosyltransferase family 2
SM2	*folC*	G421S	Dihydrofolate synthase/folylpolyglutamate synthase
SM3	*dedD*	Q35Stop	Cell division protein
SM4	*rpoA*	N294H	RNA polymerase alpha-subunit
SM5	*rpoA*	N294H	RNA polymerase alpha-subunit
SM7	*rpoA*	N294H	RNA polymerase alpha-subunit
SM8	*dedD*	V1L	Cell division protein
SM8	CO715_12235	V149G	Capsule synthesis, glycosyltransferase family 1
SM10	CO715_12210	D15N	Capsule synthesis, glycosyltransferase family 1
SM10	*dedD*	V1A	Cell division protein

changes in capsule glycosyltransferase genes did not produce detectable capsule. Surprisingly, several of the other suppressor mutants also failed to produce capsule, even though their mutations did not map to predicted capsule loci. This suggested that capsule production is detrimental to the growth of strain M12 in bile salts. To test this directly, growth of the wild-type M12 strain and the Δ*kpsCS* mutant were compared in LB medium or LB medium with 2% bile salts (Fig. 3D and E). Although growth in LB was identical, the Δ*kpsCS* mutant strain entered exponential phase more quickly than the wild-type strain when bile salts were present.

Our results suggested that capsule may delay the exponential growth of the bacteria when bile salts are present. This raised the possibility that capsule production may be influenced by the presence of bile salts. To test this, we grew the wild-type strain M12 in LB medium or in LB medium with 2% bile salts to examine how these conditions affected capsule synthesis. We also tested ExPEC strain CP9, which belongs to serotype K54. The chemical structures of K54 and K96 capsules are nearly identical (35). When analyzed by alcian blue staining of gels, capsule synthesis appeared to be strongly repressed in both M12 and CP9 strains when grown in bile salts (Fig. 4A). We also used flow cytometry to measure capsule attached to intact bacteria. K54 antiserum bound strongly to

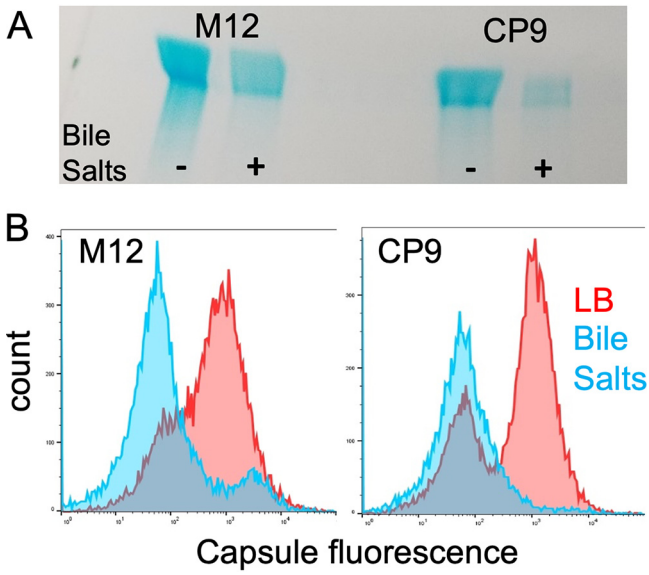

FIG 4 Growth in bile salts reduces capsule expression by strain M12 and ExPEC strain CP9. (A) Gel electrophoresis and alcian blue staining of capsule produced by M12 and CP9 grown in LB medium or LB medium containing 2% bile salts. (B) Capsule measurement by flow cytometry. The M12 Δ*kpsCS* mutant strain was used to gate for cells that reacted with K54 anticapsule serum. In both M12 and CP9, the proportion of bacteria with detectable capsule was lower when grown in bile salts than when grown in LB medium alone. Shown is a representative image of an experiment that was repeated three times.

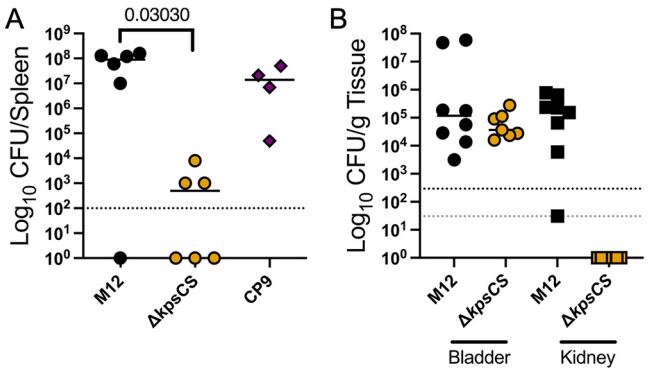

FIG 5 Virulence of mastitis-associated strain M12 in ExPEC infections depends on capsule production. (A) M12 wild-type ($n = 6$), M12 Δ*kpsCS* mutant ($n = 6$), and ExPEC CP9 ($n = 4$) strains ($\sim 5 \times 10^5$ CFU) were injected into the intraperitoneal cavity of C57BL/6 mice, and bacterial loads in the spleens were determined at 24 h. Both M12 wild-type and CP9 strains were recovered at high levels in the spleen, while the Δ*kpsCS* mutant was severely attenuated ($P = 0.0303$ by a Mann-Whitney test). The dotted line represents the limit of detection (~100 CFU). (B) Adult female Swiss-Webster mice were inoculated via transurethral catheterization and sacrificed 72 h later. Both M12 wild-type ($n = 8$) and Δ*kpsCS* ($n = 7$) strains colonized the bladders of the mice to similar levels, but in the kidney, the mutant strain was not detected ($P = 0.0003$ by a Mann-Whitney test). Black and gray dotted lines represent the limits of detection for the bladder (~293 CFU) and kidney (~30 CFU) infections, respectively.

both M12 and CP9 bacteria when grown in LB medium, but far more capsule-negative bacteria were detected when they were grown in LB medium with 2% bile salts (Fig. 4B).

Mastitis strain M12 causes urinary tract infection and sepsis in mice. MAEC strains circulate in the digestive tracts of dairy cattle and colonize mammary glands via environmental contamination. Our results suggest that capsule production may slow MAEC growth in the digestive tract where bile salts are present. Our previous work had also shown that the capsule of strain M12 contributes modestly to MG infection but is not required, which prompted us to further investigate the role of this capsule. Several MAEC strains possess similar genes for type II or type III capsules. They are required for some ExPEC strains to colonize the urinary tract and/or bloodstream. To investigate whether strain M12 could also infect these tissue sites, we used established rodent models of these human infections. First, we infected mice via intraperitoneal injection to mimic sepsis caused by ExPEC. We compared strain M12 with strain CP9, which is known to cause sepsis in this model. The wild-type M12 strain was highly virulent in these mice, which exhibited signs of terminal illness and were euthanized after 24 h. High numbers of bacteria, similar to those of strain CP9, were recovered from infected spleens when the mice were sacrificed (Fig. 5A). We also tested whether the K96 capsule was important for strain M12 to cause sepsis. In contrast to the wild-type strain, mice infected with the M12 capsule mutant strain (Δ*kpsCS*) appeared healthy, and far fewer bacteria were recovered from the spleens. We then tested whether strain M12 could infect the urinary tracts of mice. As with the intraperitoneal infections, strain M12 was very successful in the urinary tract, colonizing the bladders and kidneys of infected mice to high levels (Fig. 5B). The M12 Δ*kpsCS* mutant also efficiently colonized the bladders of the mice. However, the mutant strain was very attenuated in the kidneys, and very few bacteria were recovered (Fig. 5B).

DISCUSSION

ExPEC strains are defined not by their lineage or carriage of any particular gene but rather by their capacity to colonize specific tissue sites. However, some factors tend to be more common in specific pathotypes, including P fimbriae in uropathogenic strains (36), aerobactin siderophore and increased serum survival (*iss*) in avian pathogenic strains (37), and sialic acid capsule in neonatal meningitis *E. coli* strains (38). MAEC strains have not previously been grouped under the ExPEC umbrella, even though

some carry these virulence genes. Recent efforts to better understand the genomes of MAEC strains have uncovered certain features that tend to be associated with this group of bacteria (8, 9, 11, 39, 40). For example, MAEC strains are more likely than other strains to express the ferric dicitrate receptor, and this improves bacterial growth in milk and lactating mammary glands (12). It is now clear that all *E. coli* strains are not equally capable of causing mastitis and that the disease outcome depends greatly on the nature of the infecting strain rather than simply on host factors alone. For instance, strains that cause persistent mastitis tend to be more motile and more resistant to complement than those that cause transient disease (40). Our results indicate that some classical ExPEC factors such as capsule and metal-scavenging systems may also enhance the growth of some strains in MGs. More frequent systemic spread or severe tissue damage would logically follow from prolific growth in the udder, and these ExPEC virulence factors may also contribute to bacterial survival beyond the MG.

Our interest in the role of the *znuABC* system stemmed from our previous TnSeq data that indicated that it is required for M12 fitness in MGs and during *Galleria mellonella* infections (12), suggesting that the bacteria experience zinc starvation in these environments. In MGs, the S100 family of EF-hand proteins is thought to limit the availability of transition metals (reviewed in reference 41) Psoriasin (S100A7), a zinc-binding protein produced by keratinocytes, is found abundantly in mammary epithelia and is capable of inhibiting *E. coli* growth *in vitro* or in skin lesions (42, 43). Neutrophils also infiltrate infected MGs quickly after infection and are critical for containing the bacteria and limiting tissue damage (44). They release calprotectin (S100A8 and S100A9) to significantly limit extracellular zinc in addition to other metals (45). Resident macrophages also engulf MAEC, where the bacteria may also face toxic levels of excess zinc (46). Thus, systems to maintain zinc homeostasis may be key fitness determinants for these bacteria.

We were surprised to find that bile salts delayed the growth of the M12 Δ*znuABC* mutant. Mutants lacking these genes have been made in other *E. coli* strain backgrounds, but no similar growth defects on MacConkey agar were reported (28, 33). Whether *znuABC* mutations in other strain backgrounds confer growth defects in bile salts deserves further investigation. Bile concentrations reach levels of between 0.2 and 2.0% in the small intestine (47). Due to the detergent properties of bile, it is capable of disrupting bacterial membranes and crossing into the cell, which can induce DNA damage and oxidative stress (48, 49). Bacteria employ multiple tactics to combat the disruptive effects of bile, including outer membrane modifications, efflux pumps, and DNA repair systems (47). It is likely that variations of cell envelope features such as capsule or lipopolysaccharide greatly influence the sensitivity of different strains to bile salt stress. This is supported by our finding that the loss of capsule enabled the M12 Δ*znuABC* mutant to grow in the presence of bile salts. The K96 capsule made by M12 consists of glucuronic acid and rhamnose and has a strong negative charge (35). It is conceivable that this capsule interferes with zinc cation movement into the cells when the high-affinity Znu system is not present and zinc is not readily available. Our results indicate that bile salts may confer this type of stress, reducing the amount of zinc available for bacterial growth.

To our knowledge, there are no reports of bile salts specifically conferring zinc stress in other bacteria. However, the secondary bile acid deoxycholic acid can form organometallic complexes with zinc and other heavy metals, which may make them unavailable for import, potentially affecting gene expression in many enteric bacteria (50–53). *E. coli* O157:H7 is reported to increase the expression of iron acquisition genes when exposed to bile but repress key virulence genes, including Shiga toxin and the locus of enterocyte effacement (54). We found that both the MAEC strain M12 and the human sepsis strain CP9 repress the production of capsule in the presence of bile salts. These bacteria may benefit from repressing virulence factors while transiting through the small intestine until the appropriate target sites are reached: the colon in the case of EHEC and extraintestinal tissues for M12 and CP9. The relevant sensing mechanisms and the level at which regulation is achieved remain to be identified. Furthermore, it is not yet clear whether the influence of bile salts on capsule production occurs through

its effect on zinc availability or via sensing bile salts directly. Zinc regulation of capsule production in bacteria is not without precedent: in *Streptococcus pyogenes*, high levels of zinc such as those found within neutrophil phagosomes inhibit the phosphoglucomutase enzyme needed to initiate capsule synthesis (55).

ExPEC capsules impact pathogenesis in nonuniform ways. More than 100 distinct capsule types have been described, which are categorized into four groups based on gene organization and mode of assembly and export (56). Strain CP9 produces a K54 capsule belonging to group III that has a strong effect on virulence. CP9 mutants lacking this capsule were highly attenuated in mouse bloodstream infections and in a rat model of abscess formation (57). However, the loss of the K54 capsule did not have a measurable effect on the ability of strain CP9 to colonize mouse bladders or kidneys during urinary tract infections (58). Most uropathogenic ExPEC strains produce capsule belonging to group II. For instance, strain CFT073 produces a K2 capsule that was shown to have a modest effect on promoting colonization in mouse bladders (59, 60) and a more important role in kidneys (59). In uropathogenic *E. coli* (UPEC) strain 536, the production of the K15 capsule was proven to contribute dramatically to urovirulence in neonatal mice (61). In these studies, mortality of the mice was measured and not bacterial loads in the bladder or kidneys, so it is unclear at which stage colonization was affected.

We have shown that an MAEC strain, M12, colonizes both bladders and kidneys during experimental urinary tract infections and causes lethal sepsis in mice (Fig. 5). In both cases, the production of K96 capsule was a critical virulence determinant for the colonization of kidneys and spleens. Previously, our group showed that this mutant is also highly attenuated in *G. mellonella* (12). It is interesting that the capsules of strains M12 and CP9 are identical, but in CP9, the capsule is dispensable for kidney infection via the urinary tract (58), whereas our results show that it is required in strain M12. The absence of the K96 capsule of M12 did not detectably alter bladder colonization (Fig. 5) and had a moderate effect in MGs. The exact function of the M12 capsule in these specific tissues is not yet understood. ExPEC capsules may have a role in avoiding killing by phagocytes (62). The capsules of some ExPEC strains are required for serum resistance, while in other strains, the loss of capsule has no effect (63–66). ExPEC capsule can also help in the establishment of intracellular bacterial communities within epithelial cells of the urinary tract (67). The fact that some MAEC strains carry capsule and other ExPEC virulence genes has been noted previously, but ours is the first study to show that an MAEC strain is capable of causing urinary tract infection and sepsis in established models of human disease. Nonpathogens may carry ExPEC virulence factors because they promote intestinal colonization rather than infection, depending on the strain background. However, as we have shown here, some MAEC strains may not be purely commensals or accidental pathogens but rather versatile organisms fully capable of causing human illness in addition to bovine mastitis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strain CP9 is a human bloodstream isolate that has been previously described in detail (68). *E. coli* strain M12 and the M12 Δ*kpsCS* mutant were previously characterized (12). *E. coli* strains were routinely grown in Luria-Bertani (LB) medium at 37°C. To select for mutant strains or carriage of plasmids, growth medium was supplemented with chloramphenicol (10 μg/ml) or ampicillin (100 μg/ml) as appropriate. For milk cultures, whole unpasteurized cow's milk was obtained from a local supplier and used immediately or stored at −80°C until use. Growth curves were generated using a Bioscreen C system (Labsystems Oy, Helsinki, Finland). Bacterial cultures were standardized to 5×10^4 CFU/ml, and the absorbance at 600 nm was measured every 15 min with continuous shaking at 37°C. LB growth medium was supplemented with 2% bile salts, 2 μg/ml polymyxin B, 5% SDS, EDTA, zinc sulfate, manganese sulfate, or copper sulfate as appropriate. All chemicals were purchased from Sigma-Aldrich. For competition assays during growth in milk, equal ratios of M12 and Δ*znuABC* mutant bacteria were inoculated into whole unpasteurized cow's milk and incubated at 37°C for 8 h. CFU ratios of the input and output for M12 and the Δ*znuABC* mutant, determined by plate counts, were used to calculate a competition index.

Deletion and complementation of *znuABC*. Mutation of *znuABC* was performed via lambda red recombination in strain M12 carrying the plasmid pKD46 (69). A PCR product was created using pRE112 (70) as a template to amplify the chloramphenicol acetyltransferase gene, with 50-bp overhangs homologous to the 5′ end of *znuB* or the 3′ end of *znuA*. Bacteria expressing the recombinase enzymes were

electroporated with 500 ng of purified DNA, and potential mutants were selected by plating on LB agar containing chloramphenicol. Potential mutants were then screened by PCR using primers flanking the recombination site and also primers internal to the *znuB* and *znuA* genes. The correct Δ*znuABC* mutation and the absence of other SNPs were also confirmed by whole-genome sequencing (Microbial Genome Sequencing Center, University of Pittsburgh). To complement the mutation, the *znuABC* genes were PCR amplified, inserted into the pACYC184 plasmid by overlapping-extension PCR, and transformed into *E. coli* DH5α to create plasmid pMO3.

Suppressor mutant sequencing, assembly, and annotation. Total DNA was isolated from suppressor mutants using a ZR fungal/bacterial DNA miniprep kit (Zymo Research). DNA sequencing libraries were prepared using the Illumina Nextera DNA library prep kit with modifications (71). DNA libraries were sequenced by Genewiz, Inc. (South Plainfield, NJ), and Illumina paired-end reads of 150 bp were generated on a MiSeq version 2 sequencer. Contigs, annotations, and data from SNP analyses were compiled using EnteroBase (72, 73).

Capsule isolation and staining. Capsule production was visualized as previously described (74). Briefly, bacteria were pelleted from 1 ml of saturated cultures grown overnight in LB broth. The bacterial cells were then resuspended in 50 μl of phosphate-buffered saline (PBS) and heated to 55°C for 30 min. The capsule material was analyzed by electrophoresis on 10% SDS-polyacrylamide gels and staining with 0.125% alcian blue dye in 40% ethanol–5% acetic acid.

Flow cytometry. Bacteria were grown in LB broth or LB broth supplemented with 2% bile salts for 24 h. Saturated cultures grown overnight were diluted 1:100 in 0.1% bovine serum albumin (BSA)–PBS with undiluted anti-K54 rabbit antisera (SSI Diagnostica) and incubated for 30 min on ice. Samples were washed with 0.1% BSA–PBS and stained with a goat anti-rabbit IgG(H+L) secondary antibody (Ab), Alexa Fluor Plus 594 (Invitrogen), in the dark for 30 min. The fluorescence of individual bacterial cells was measured using a BD Accuri C6 flow cytometer, and histograms were generated with FlowJo software. Negative-control samples contained bacteria with the secondary Ab only or without primary and secondary Abs.

Ethics statement. Mouse experiments were performed in accordance with the recommendations found in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (75). The protocol was reviewed and approved by the Institutional Animal Care and Use Committees of Brigham Young University and the University of Utah.

Mouse infections. (i) Mouse mammary gland infections. Lactating C57BL/6 mice between 9 and 12 weeks of age and 10 to 11 days postpartum were infected as previously described (12). Briefly, a 50-μl volume of bacteria containing 250 CFU of both wild-type M12 and the Δ*znuABC* mutant strain in PBS was injected directly through the teat canal into the ductal network of the 4th left and 4th right mammary glands of 3 mice using a 33-gauge needle with a beveled end. Pups were removed for 1 to 2 h after injections and were then returned. Mice were euthanized after 24 h, and mammary gland tissue was harvested. Bacterial loads were determined by homogenizing the tissue in 1 ml of PBS, performing serial dilutions, and plating on selective agar. CFU ratios of the input and output for M12 and the Δ*znuABC* mutant, determined by plate counts, were used to calculate a competition index.

(ii) Mouse intraperitoneal infections. Equal numbers of male and female C57BL/6 mice between 9 and 12 weeks of age were used for all infections. Bacteria were grown overnight in LB medium, subcultured to an absorbance of 1.0 at 600 nm, and serially diluted in PBS. A 200-μl volume of bacteria containing 5×10^5 CFU in PBS was injected directly into the intraperitoneal cavity using a 27 1/2-gauge needle. The concentration of each inoculum was determined by serial dilution and colony counting after 24 h of growth on LB agar plates. Mice were monitored for 24 h, and spleens were harvested. Bacterial loads were determined by homogenizing entire spleens in 1 ml of PBS and performing serial dilutions and colony counts.

(iii) Mouse urinary tract infections. Adult (~8-week-old) female Swiss-Webster mice (Charles River) were used in all experiments. Bacteria were grown statically from frozen stocks in 20 ml M9 minimum medium for 24 h at 37°C. Bacteria were pelleted at 8,000 relative centrifugal force (RCF) for 8 min and resuspended in 3 ml sterile PBS. Mice were inoculated with a 50-μl volume (5×10^8 CFU) via transurethral catheterization and sacrificed 3 days later. Kidneys and bladders were weighed and homogenized, and CFU were determined by serial dilution and plating.

Statistical analyses. All mouse infection data were analyzed using GraphPad Prism 5.0. The statistical tests performed as well as the significance values are indicated in the individual figure legends.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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